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Award Number: DAMD17-01-1-0361

TITLE: Effects of Moderate Aerobic Exercise Combined with Caloric Restriction on Circulating Estrogens and IGF-I in Premenopausal Women

PRINCIPAL INVESTIGATOR: Nancy I. Williams, Sc.D.

CONTRACTING ORGANIZATION: The Pennsylvania State University
University Park, Pennsylvania 16802-7000

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PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

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**13. ABSTRACT (Maximum 200 Words)**

The proposal entitled "Effects of moderate aerobic exercise combined with caloric restriction on circulating estrogens and IGF-I in premenopausal women" will provide important scientific contributions with respect to the primary prevention of breast cancer in women. Specifically, this study will examine potential mechanisms relating to the role of physical activity in the reduction of the risk of breast cancer by testing whether moderate aerobic exercise can reduce the levels of two hormonal biomarkers, circulating estrogens and insulin-like growth factor I (IGF-I). as expected caloric restriction combined with aerobic exercise training occurring 4 times per week has resulted in increases in aerobic capacity, weight loss ranging from 2.5-7.5 kg and loss of body fat raging form 3% to 7% over four months. Results for T3, IGF-1, and estrone look prmising, although the number of subjects who have completed the study thus far is too low to test these changes statistically. Compliance to our dietary and exercise protocols has been excellent, with subjects completed 3.8/4 workouts per week, and meeting or exceeding their caloric intake goals. In conclusion, we are making good progress toward completion of this study.

| 14. SUBJECT TERMS | 15. NUMBER OF PAGES |
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| Breast cancer, menstrual cycle, IGF-1, estradiol, exercise | 26 |
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| 17. SECURITY CLASSIFICATION OF REPORT | 18. SECURITY CLASSIFICATION OF THIS PAGE | 19. SECURITY CLASSIFICATION OF ABSTRACT | 20. LIMITATION OF ABSTRACT |
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## Table of Contents

# INTRODUCTION

This proposal entitled " Effects of moderate aerobic exercise combined with caloric restriction on circulating estrogens and IGF-I in premenopausal women" will provide important scientific contributions with respect to the primary prevention of breast cancer in women. Specifically, this study will examine potential mechanisms relating to the role of physical activity in the reduction of the risk of breast cancer by testing whether moderate aerobic exercise can reduced the levels of two hormonal biomarkers, circulating estrogens and insulin-like growth factor I (IGF-I). Since elevated levels of both of these hormones have been associated with an increased risk of breast cancer, and because exercise may modulate circulating levels, we wish to extend previous findings from epidemiological and cross-sectional studies by performing a tightly controlled, prospective clinical study that addresses previously unanswered questions related to the role of exercise in the modulation of estrogen and IGF-I. Although previous studies have shown that negative energy balance, and not other stressful aspects of physical exercise, can modulate reproductive function and therefore circulating estrogen levels, no studies to date have determined the magnitude of energy deficit required for these changes during long-term training, and no studies have attempted to differentiate between the exercise-induced changes in ovarian versus adipose sources of circulating estrogens. Since both estradiol (ovarian) and estrone (adipose tissue) are biologically active, and because the importance of estrone as a risk factor increases with age and adiposity, it is important to consider the degree to which exercise which creates a negative energy balance affects both of these sources of circulating estrogens. Circulating levels of IGF-I correlate with breast cancer risk, yet studies examining the responses of this hormone and its binding proteins to chronic exercise are lacking. Since IGF-I levels are very sensitive to nutritional status, previously reported stimulatory effects of exercise on IGF-I can be overridden if exercise is performed in the face of negative energy balance. In this regard, exercise that promotes weight loss can be viewed as a way to reduced levels of IGF-I, and therefore potentially reduce the risk of breast cancers. To date, no studies have addressed whether a program of moderate aerobic exercise and dietary restriction producing a negative energy balance that is carried out over a long duration will significantly alter IGF-I levels. Further the degree to which these levels might be altered in individuals of differing energy stores has not been addressed. Metabolic energy availability is an important contributing factor in the development of reproductive cancers. However, current methods for assessing energy availability, which include anthropometric measures, calculations of energy balance, evaluation of various serum and urinary biomarkers are prone to measurement error, not sensitive to alterations in energy availability, and are sometimes affected by disease states. The current project centers on the introduction of a novel approach to estimating energy status by measuring metabolic hormones in plasma, insulin, IGF-I, IGFBP-1 and leptin. Recently, dried blood spot (DBS) sample collection techniques have allowed for endocrine based population studies examining a wide variety of ecological factors that contribute to variation in human reproduction. In order to use the proposed method of energy status assessment in large population-based applications, such as those addressing the role of physical activity and or diet in the risk of breast cancer, the battery of metabolic hormones that comprise the proposed method must be amenable to collection and assays. Although the DBS technique has been partially validated for some hormonal assays, it has not yet been properly validated for insulin, IGF-I, IGFBP-1 and leptin, and it is unclear whether the technique is responsive to physiological changes of these compounds. Therefore, the current work calls for the validation of the DBS sampling technique for these assays under physiological conditions.The proposed studies will yield new and important information regarding the degree to which an exercise and diet program that results in an energy deficit will reduce the risk of breast cancer.

# BODY

Study Design: The study utilizes a prospective, randomized design that tests the effects of a moderate exercise program (4X/wk; 4 months) combined with moderate dietary restriction that results in an average daily energy deficit of -20%-30% kcals (Figure 1). Previously sedentary, eumenorrheic women aged 25-40 years will be assigned to exercise or control groups. Both normal weight (BMI 21-25 kg/m$^2$) and overweight (BMI 26-30 kg/m$^2$) will be assigned to either exercise or control (no exercise, no dietary restriction) groups; 4 groups, n=15 each group. Subjects will be studied for a total of six menstrual cycles, i.e., 2 control followed by 4 cycles with training and dietary restriction.

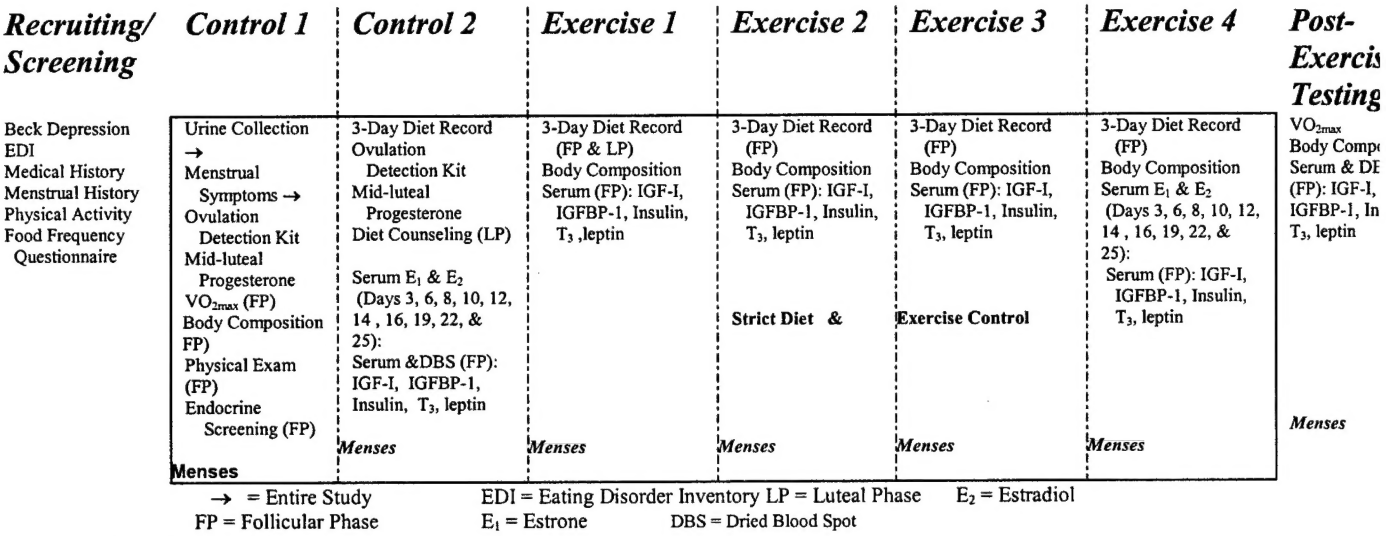



**Figure 1. Study Design**

## Progress According to the Approved Statement of Work:

(See previous Annual Summary for 2001-2002)

**********

*Proposed* Month 13-16

1. Repeat steps above for year 2 recruiting and beginning testing (n=15 in each of 4 groups)
2. Perform assays on metabolic hormones in serum
3. Send serum and blood spot samples from year 1 subjects to DSL
4. Perform urinary assays on LH, E3G, PdG

*Actual Month 13, September, 2003*- We continued aliquotting and processing urine samples and data entry.

*Actual Month 14, October, 2002* -completed data entry and are beginning preliminary data analysis; began assaying urine for E1G and PDG; beginning to assay metabolic hormones, i.e., insulin, leptin, T3, and IGF-I; preparing data reports for subjects that completed the study, and getting ready to recruit for Year 2.

*Actual Month 15, November, 2002*-Finished sending out data reports for all nine subjects that completed; held off on recruiting until Spring due to upcoming holiday season; continued assaying urine, but encountered difficulties with performance of research technician assigned assay duties

*Proposed* Months 16-24

1. Continue year 2 recruitment efforts only if necessary

2. Continue year 2 subject screening/initial testing
3. Complete year 2 subject exercise training/experimental testing
4. Perform urinary assays on LH, E1G, PdG

*Actual Month 16, December, 2002*- Held off running urinary assays, re-assigned duties of research technician, planned for new recruitment strategy
*Actual Month 17, January, 2003*-Placed ad for volunteers in local newspaper, and began "rolling recruitment strategy", by continuing to place ads and posting weekly fliers on campus. This was due to low enrollment. We also increased the age of enrollment to 40 years, again to increase study numbers. This increase will not affect the scientific underpinnings of the study.
*Actual Month 18, February, 2003*- Enrollment continuing, several subjects began study
*Actual Month 19, March, 2003*- Enrollment continuing, several subjects began study
*Actual Month 20, April, 2003* Enrollment continuing, several subjects began study; having enlisted new technical help, began urinary assays for subjects that had completed study
*Actual Month 21, May, 2003* Enrollment continuing, several subjects began study; continued assaying urine for reproductive hormones, began assaying metabolic hormones and serum estradiol and estrone
*Actual Month 22, June, 2003*- Enrollment continuing, several subjects began study; completed urinary assays of E1G and PDG; began assays for T3
*Actual Month 23, July, 2003*- Enrollment continuing, several subjects began study; continued assays for T3
*Actual Month 24, August, 2003;* Enrollment continuing, several subjects began study; continued assays for T3; began assays for IGF-1
*Actual Month 25, September, 2003*- Enrollment increased dramatically, fourfold increase in enrollment; assays completed, T3, and continued for IGF-1; arrangements made with Salimetrics Laboratory, University Park, PA to develop blood spot assays for Leptin, T3, and IGF-I (See Appendix); begun assays on urinary LH to document LH surges.

**Preliminary Results From Years 1 and 2:**

**Subject Recruitment:** We have accumulated over 249 contacts since September of last year. We have recently stepped up recruitment efforts and plan to enroll 55-60 new subjects by January, 2004. This will allow us to reach our target initial enrollment of 60, but we may need to extend the time frame of the study if sign-ups slow down towards the holidays. Forty-six women started the study and 19 have dropped out for the following reasons: 3 for menstrual abnormality, 5 medical, 10 self (time, intervention, etc.), and 1 noncompliant. The forty-six women described their ethnicity as the following: 32 Caucasian, 8 Asian, 3 African-American and 2 other. Ten women have completed the study, fourteen women are more than half-way through the study at this point in time.

Table 1. Initial characteristics of subjects completed and currently enrolled

| Group | Age (yrs) | Weight (kg) | Height (cm) | BMI ($kg/m^2$) | % Fat | $VO_2$ max (ml/kg/min) |
|---|---|---|---|---|---|---|
| Light Conditioning/Low BMI | 32 ± 4 | 63 ± 3 | 167 ± 6 | 23 ± 2 | 29 ± 1 | 31 ± 1 |
| Light Conditioning/ High BMI | 33 ± 5 | 68 ± 2 | 156 ± 6 | 28 ± 2 | 35 ± 5 | 25 ± 3 |

| | | | | | | |
|---|---|---|---|---|---|---|
| Exercising/ Low BMI | 31 ± 4 | 62 ± 6 | 165 ± 6 | 23 ± 2 | 27 ± 5 | 32 ± 5 |
| Exercising/ High BMI | 30 ± 6 | 77 ± 8 | 166 ± 6 | 28 ± 2 | 37 ± 3 | 29 ± 7 |

Values are mean +/- SD

**Results From Subjects Completing the Study:** Subjects met our initial targets for weight, age, BMI and fitness levels. Average menstrual cycle length was 29.7 ± 5 days, and did not change significantly in either Low or High BMI group. Aerobic exercise training was 4 times per week for four consecutive menstrual cycles at 76± 3 % of maximum heart rate for 40-60 minutes, resulting in an average of 24% increase in aerobic capacity as defined by $VO_2$ max (32.6 ± 4.7 to 42.0 ± 10 ml/kg/min; $P < 0.05$ pre vs post). Dietary intake was successfully reduced using the food exchange system (Low BMI= 1778 ± 234 to 1230 ± 139 kcals; High BMI =2345 ± 187 to 1350 ± 284 kcals; $P< 0.05$ pre vs post in both groups). The combination of moderate exercise and diet produced significant weight loss in both groups (Low BMI -3.3 %; High BMI -7.6% $P< 0.05$, Figure 3). Significant changes in percent body fat occurred in both groups (Low BMI 32 ±4 % to 25 ± 8 % ; High BMI 39 ± 5 to 32 ± 6 %: $P < 0.05$).

**Body Weight Changes of Subjects Currently Exercising:** Figures 1-4 illustrate the changes we are seeing in body weight in our experimental groups that are currently exercising. These changes in body weight represent an estimated 30% increase in energy expenditure for the "exercising groups", combined with a 25% reduction in caloric intake. The exercise completed by the "light conditioning" group represents an estimated 10% increase in energy expenditure, and no changes in caloric intake. All subjects meet every other week with the nutritionist to go over dietary records and review educational modules for that week.

**Urinary Assays:** We have completed urinary E1G and PDG for 9 subjects thus far and are now confirming ovulation day in these cycles by assaying urinary LH. We expect to complete these assays within the next two months, and then continue running them in batches as subjects complete the study. Preliminary results suggest no changes in cycle length, follicular phase length, luteal phase length with exercise, but a trend toward a reduction in the magnitude of the pre-ovulatory estradiol peak. Figure 5 shows representative data for subject D-001, and illustrates this reduction in the estradiol peak.

**Metabolic Hormone Assays:** We have completed assays for subjects who have completed the study for T3, and are in the middle of IGF-1 and leptin. We plan to run insulin and IGFBP-1 in the next three months, and continue with all these assays as individuals finish the study. We will run them in batches of 3-4 subjects at a time. Preliminary results indicate that IGF-1 is reduced with weight loss. Figures 6-8 illustrate this decrease in during the Control 2 month, and then during Exercise 4, in two out of three subjects who lost 3.2 to 7.5 kg

**Serum estradiol and estrone:** We have completed these assays for 9 subjects, and have them up and running to complete as subjects finish the study. We will run them in batches of 3-4 subjects at a time. Figures 9-12 changes in serum estrone, estradiol, and urinary estrone-1-glucuronide.

**Blood Spot Assays:** We had technical/administrative problems with our contracted source for the development of the blood spot assays, Diagnostic Systems Laboratory (see information in Appendix). They discontinued the blood spot assays. ZRT Laboratory was recommended. The PI contacted ZRT Laboratory and was unsatisfied with their customer support and knowledge base regarding quality control of the assays. The PI then re-contacted Doug Granger of Salimetrics at Penn State, and we now have a contract with them to develop the assays for leptin, T3, and IGF-1. We chose these hormones because they will be the best indicators of energy

availability, and therefore correlate the best with changes in body weight and estradiol and estrone. We should get results from them in six months time from this point forward.
**Personnel/Technical Issues**: Our progress has been delayed somewhat due to personnel issues relating to poor performance on the part of a research technician who is no longer with us. Additionally, our project coordinator is out on maternity leave but will return in four weeks.
**Overall Results from Years 1 and 2:** All aspects of the study are up and running. Preliminary analyses indicate support for the hypotheses that estrone and IGF-1 will decrease with our exercise and diet intervention. Assay development using the blood spot technique is progressing, and thus we will be able to test the validity of this method for two newly developed assays, i.e., T3 and IGF-1, both of which, in our hands, will be very useful for doing field work and or obtaining samples from subjects outside of our immediate geographical area. Future grant proposals, and follow-up studies will make use of these techniques.

## KEY ACCOMPLISHMENTS

This is an ongoing study, so preliminary publication of the data is not feasible.

## REPORTABLE OUTCOMES

The following outcomes have occurred since Dr. Williams received BCRP funding.

**Published Manuscripts:**

**Williams, N.I.**, Caston-Balderrama, A.L. Helmreich, D.L., Parfitt, D.B., Nosbisch C, Cameron, J.L. Longitudinal changes in reproductive hormones and menstrual cyclicity in cynomolgus monkeys during strenuous exercise training: rapid transition to exercise-induced amenorrhea *Endocrinology* 142: 2381-2389, 2001

**Williams N.I.,** DL Helmreich DL, DB Parfitt, Caston-Balderrama AL, JL Cameron. Evidence for a causal role of low energy availability in the induction of menstrual cycle disturbances during strenuous exercise training. *J Clin Endocrinol Metab* 86: 5184-5193, 2001

Miles MP, Mackinnon LT, Grove DS, **Williams NI**, Bush JA, Marx JO, Kraemer WJ, Mastro AM. The relationship of natural killer cell counts, perforin mRNA and CD2 expression to post-exercise natural killer cell activity in humans. *Acta Physiol Scand* 174: 1-9, 2002.

McConnell HJ, KA O'Connor, E Brindle, and **NI Williams**. Validity of methods for analyzing urinary steroid data to detect ovulation in athletes. *Med. Sci. Sports Exerc,* 34(11): 1836-1844, 2002

Whipple TJ, Petit Moira, Sharkey N, Demers L, **Williams NI**. Leptin and the skeleton. *Clin. Endocrinol.* 57: 701-711, 2002.

**Williams, NI.** Experimental disruptions of the menstrual cycle: Lessons from long-term prospective studies. *Med Sci Sports Exerc* 35 (8): 1564-1572, 2003.

**Manuscripts in Review**

**Williams, NI,** Flecker KL, McConnell. Weight and Diet Concerns in Female Athletes: Association with Menstrual Cycle Length (submitted to *Int J Sports Nut Exerc Metab*, September, 2002)

Whipple TJ, Le B, Demers LM, Petit M, Sharkey N, and **Williams NI**. Acute effects of moderate intensity resistance exercise on bone cell activity (submitted to the *International Journal of Sports Medicine*, 2003).

McConnell HJ, Gardner JK, Frye BR, Snook ML, Schuchert MK, Richard EL, and **Williams NI.** Basal Ghrelin is Sensitive to Changes in Body Composition and Metabolic Rate during a Diet and Exercise Program in Normal Weight Young Women. (Submitted to *J. Clin. Endocrinol. Metab.* as a Rapid Communication, 2003).

**Williams, N.I.** , Berga S.L., and Cameron, J.L. Synergism of multiple sub-threshold stressors: effects of diet, exercise, and psychosocial stress on menstrual cyclicity. (*submitted to Nature Medicine, October, 2003)*

Abstracts

Mastro AM, **Williams NI**, Kraemer WJ, Orsega-Smith EM, Perry MD, Dixon RH, Bleznak AD, Underwood J. Exercise, quality of life, and the recovery of CD4 (+) lymphocytes following chemotherapy for breast cancer *Proceedings of the American Association for Cancer Research 92nd Annual Meeting,* New Orleans, LA , 42 : 331, March 24-28, 2001

Perry MD, Mastro AM, Orsega-Smith E, Miles MP, Kraemer WJ, **Williams NI**. Exercise training and immune function following chemotherapy for breast cancer. *Proceedings of the American College of Sports Medicine Annual Meeting*, Baltimore, MD, June 2-6, 2001

Orsega-Smith E, **Williams NI** (FACSM), Perry MD, Mastro AM, Kraemer WJ, Bleznak A, Dixon R, Underwood J. Fatigue, quality of life and physical function after chemotherapy for breast cancer. *Proceedings of the American College of Sports Medicine Annual Meeting*, Baltimore, MD, June 2-6, 2001

Galucci, AN, **Williams NI**. Physiological indicators of psychological stress prior to competitive exercise. *Proceedings of the American College of Sports Medicine Annual Meeting*, Baltimore, MD, June 2-6, 2001

McConnell HJ, O'Connor KA, Brindle E, **Williams, NI**. Assessing reproductive function in exercising women: validity of ovulation detection algorithms. *Proceedings of the Endocrine Society Annual Meeting, Abstract* #P2-408, 2001

Senior MK, **Williams NI**, McConnell HJ, Clark KC. Screening for subclinical eating disorders in female athletes: validation of an indirect interview technique. *(Presented at the 24th Annual meeting of the Mid-Atlantic Regional Chapter of the American College of Sports Medicine, Bushkill, PA, November 2-3, 2001).*

McConnell HJ, **Williams NI**, O'Connor KA, Clark KL, Putukian M. Menstrual irregularities and disordered eating in female athletes: survey vs follow-up clinical and physiological studies. *(Presented at the 24th Annual meeting of the Mid-Atlantic Regional Chapter of the American College of Sports Medicine, Bushkill, PA, November 2-3, 2001).*

Mastro AM, **Williams NI**, Ford J, Fuener K, Orsega-Smith E, Kraemer WJ, Bleznak AD, Dixon RH, Underwood J, Miles M, Wagner K. IL-6 and interferon-gamma levels following chemotherapy for breast cancer. *Proceedings of the American Association for Cancer Research Annual Meeting*, San Francisco, CA, April 6-10, 2002

Hertel J, **Williams NI**, Gribble PA, McConnell HJ, DiPasquale AA, Putukian M. Changes in risk factors of ACL injuries across the menstrual cycle: A pilot study. *Proceedings of the American College of Sports Medicine Annual Meeting*, St. Louis, MO, May 29-June 1, 2002

**Williams NI**, McConnell HM, Gardner JK, Albert AC, Cameron JL. Lifestyle factors such as exercise, caloric intake, and psychological stress: relationship to reproductive hormones and possibly the risk of breast cancer. *Era of Hope* meeting, Department of Defense Breast Cancer Research Program, Orlando, FL, September 25-28, 2002

Dougherty, K., Galucci AN, McConnell HJ, **Williams NI.** Cortisol and testosterone levels prior to competitive exercise. (Submitted for presentation at the 2003 American College of Sports Medicine Annual Meeting, San Francisco, CA, June, 2003).

**Williams NI,** McConnell HJ, Gardner JK, Cameron JL, Schuchert MK, Richard EL, Snook ML. Susceptibility of menstrual cycle to various stressors: correlation with baseline luteal progesterone levels. (Presented at the 2003 American College of Sports Medicine Annual Meeting, San Francisco, CA, June, 2003).

McConnell HJ, Schuchert MK, Gardner JK, Frye BR, **Williams NI**. Basal Ghrelin is sensitive to changes in body weight during a controlled diet and exercise program in normal weight young women. (Presented at the 2003 Endocrine Society Meeting, Philadelphia, PA, June 2003).

Whipple TJ, Le, B., Demers, L., Petit M.A., Sharkey N. **Williams, NI**. Acute Effects of Moderate Intensity Resistance Exercise on Bone Cell Activity. (Presented at Association for Bone and Mineral Research Meeting, 2003).

**Grants Applied For:**

National Institutes of Health (NIH)
1 RO1 **(Co –Principal Investigator** with Mary Jane De Souza, Univ. Toronto)
7/01/02 - 6/30/07 15%
PHS/NICHD **$ 2,433,044**
" Clinical Sequelae Exercise-Induced Hypoestrogenism"

National Insitutes of Health (NIH)
**Co-Investigator (PI is Terryl Hartman, PSU)** 4/01/04-3/31/08 20%
$2,085,448
"Female Cancer Survivors Weight and Activity Intervention"

NASA
**Co-Investigator (PI is James Pawelczyk, PSU)** 4/1/05-3/31/06 5%
$1,144,613

"Improving Orthostatic Tolerance in Women: Control of Splanchnic and Cutaneous Vascular Capacitance"

Cancer Research and Prevention Foundation
**Co-Investigator** 1/04-12/05 0%
$76,865

"Exercise and Estrogen Metabolism: Implications for Breast Cancer Prevention"

## CONCLUSIONS

We are making good progress toward the completion of this study. Preliminary examination of the data look interesting and supportive of the hypotheses put forth in the study, but statistical power is too low at this point to draw firm conclusions.

## REFERENCES

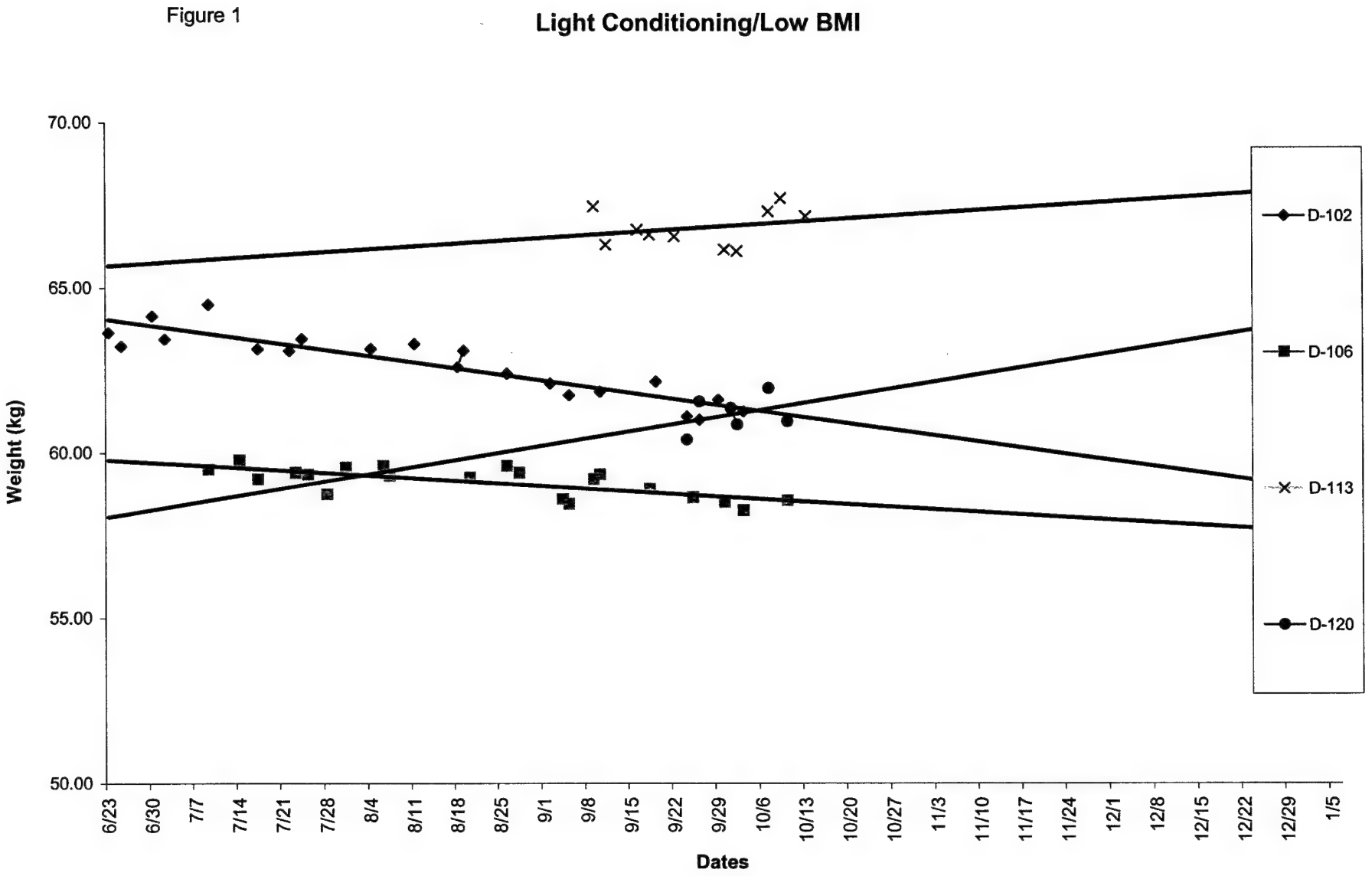
NONE

## APPENDICES

Figures 1-12

Letters to and from Salimetrics, Inc.

Figure 1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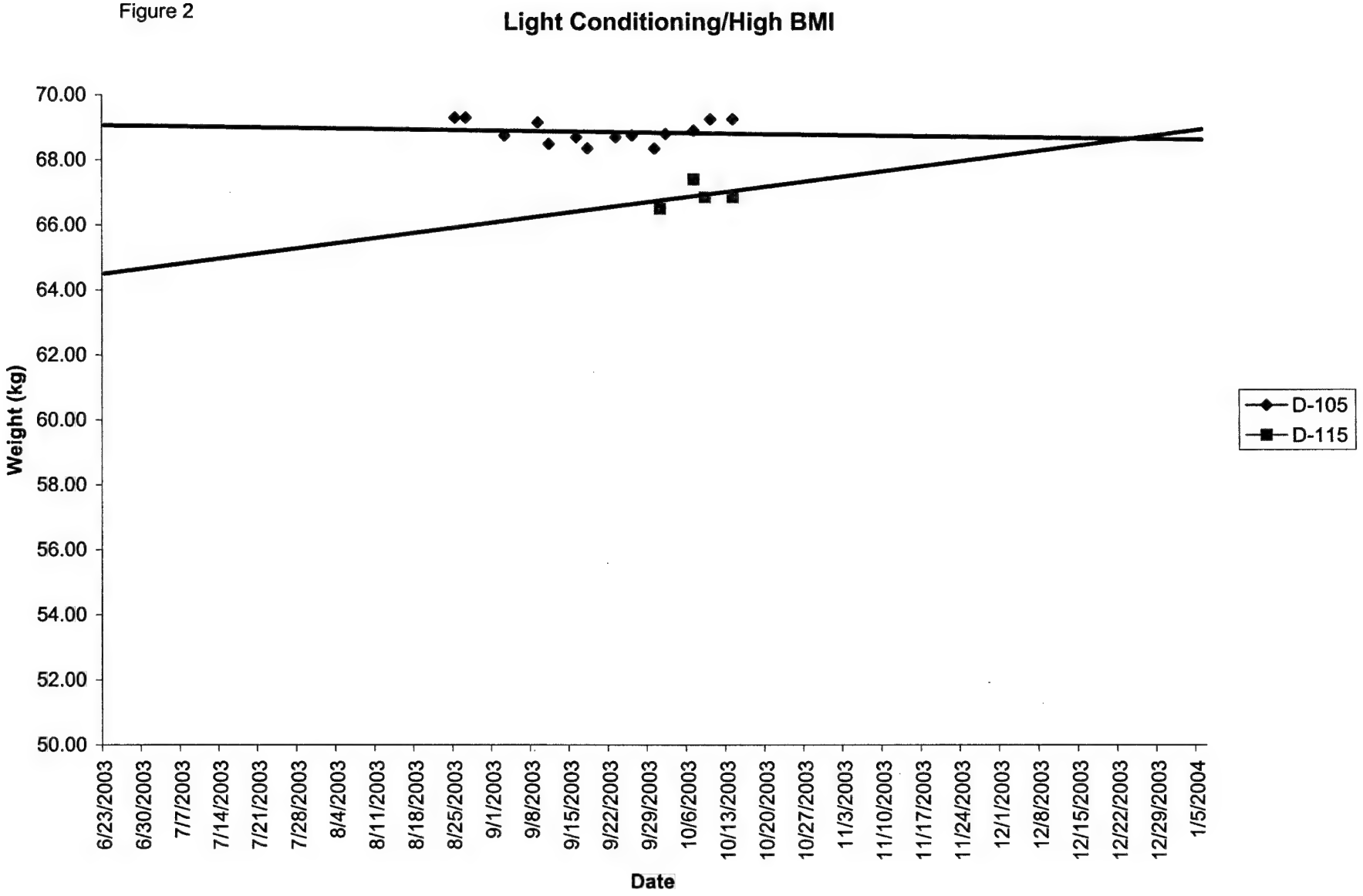
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Figure 3

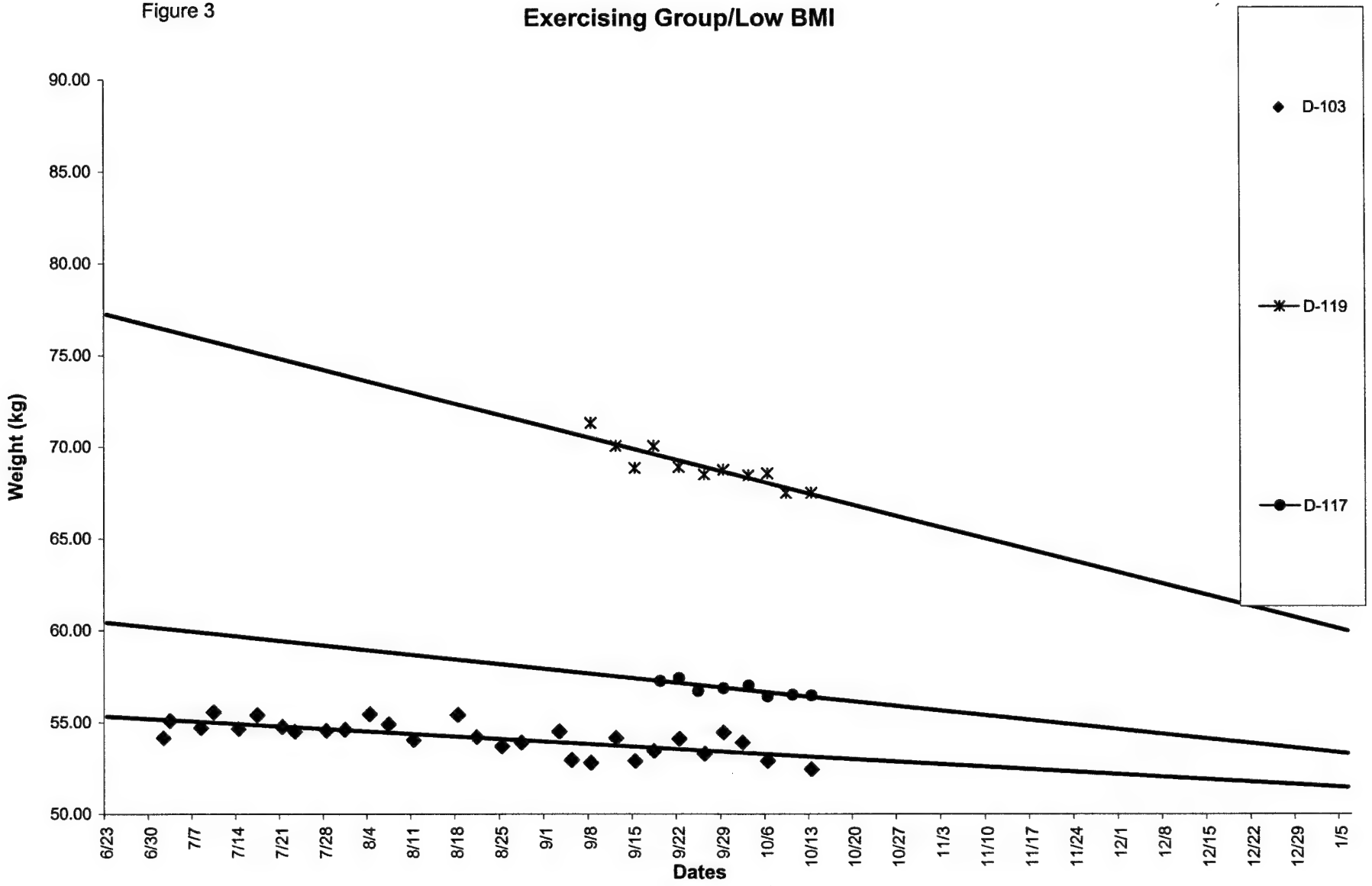
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Figure 4

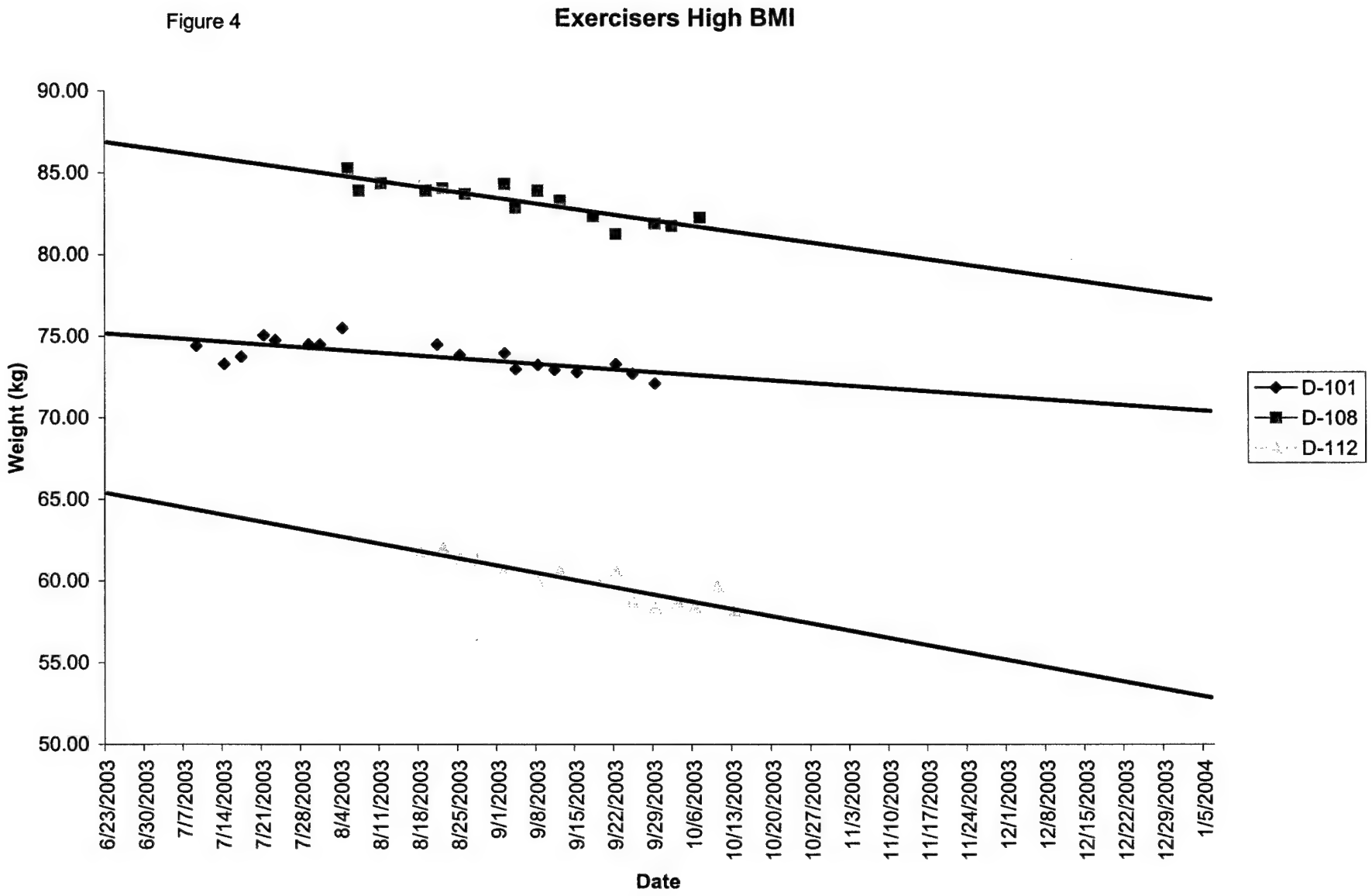
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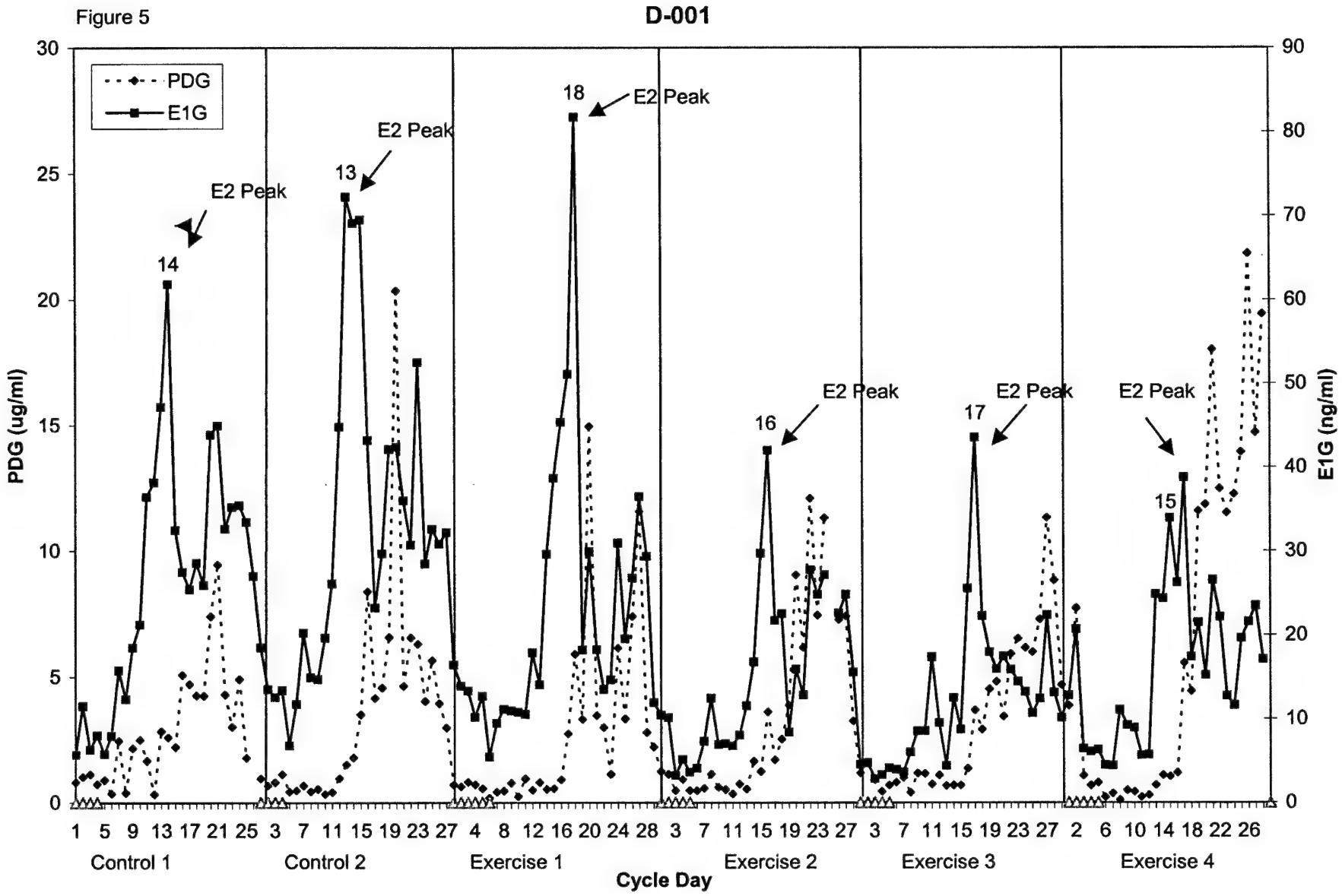
Figure 5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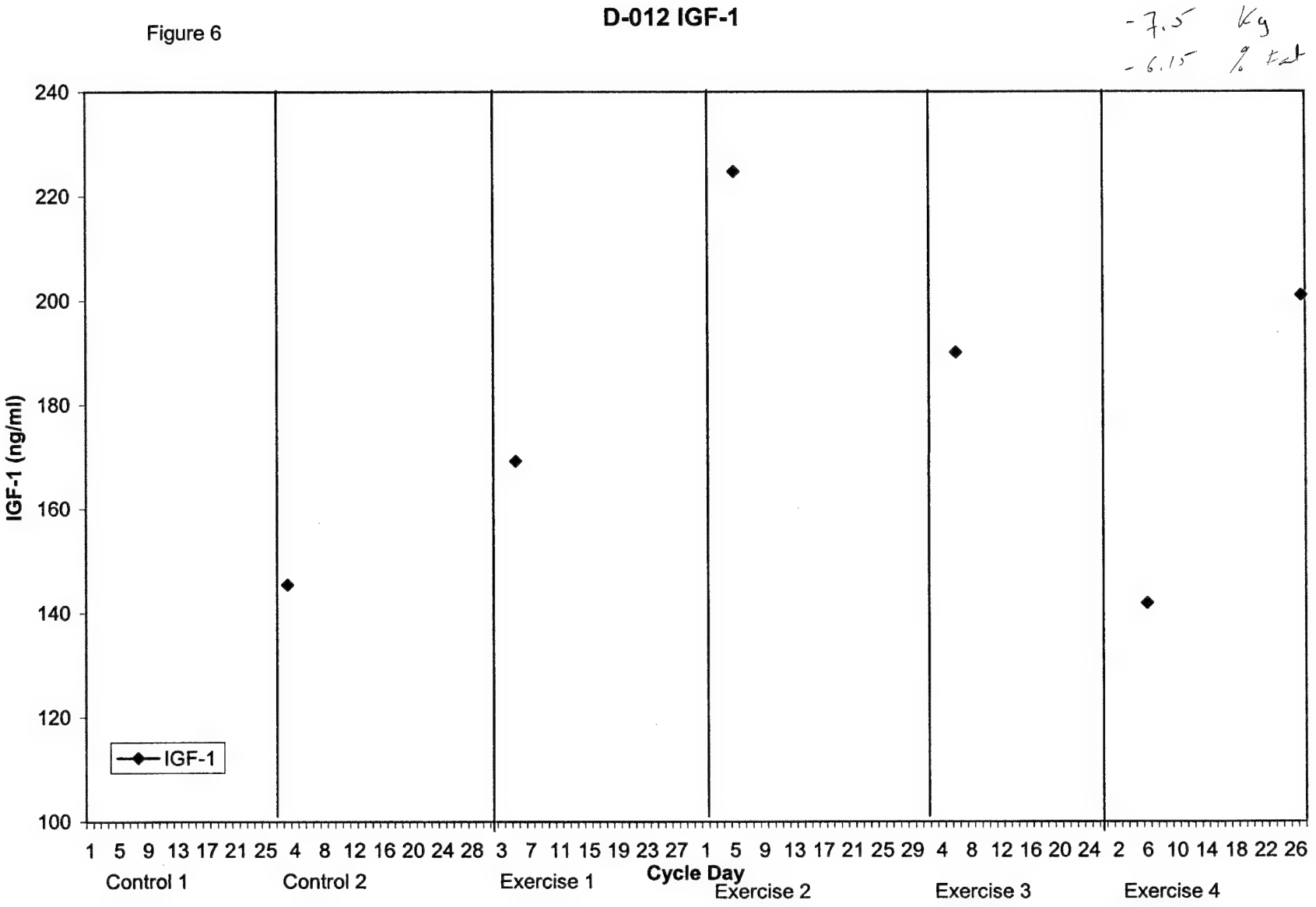
Control 1
Control 2
Exercise 1
Exercise 2
Exercise 3
Exercise 4
Cycle Day

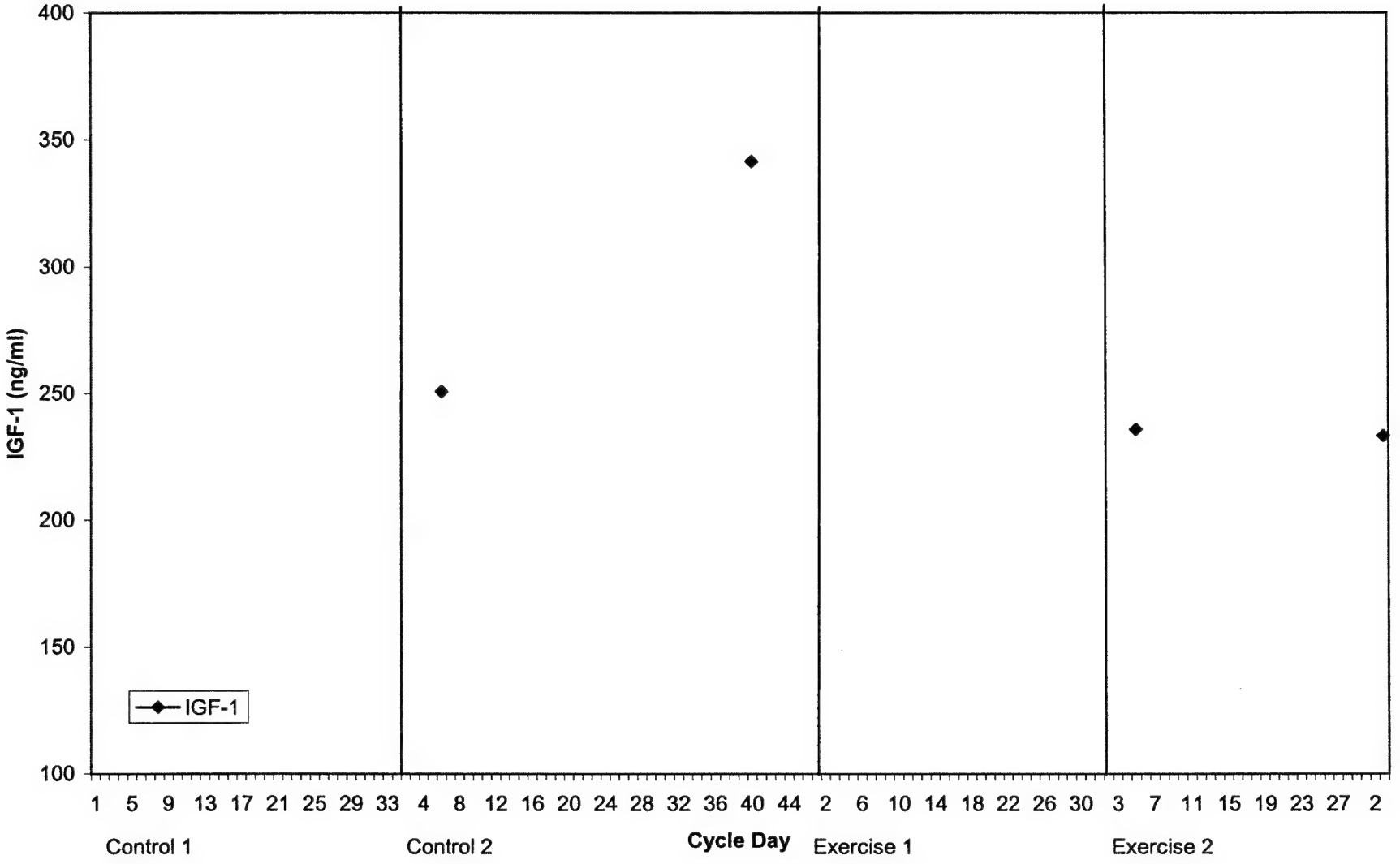
Figure 6


D-012 IGF-1
-7.5 Kg
-6.15 % fat
IGF-1 (ng/ml)
240
220
200
180
160
140
120
100
IGF-1
1 5 9 13 17 21 25 4 8 12 16 20 24 28 3 7 11 15 19 23 27 1 5 9 13 17 21 25 29 4 8 12 16 20 24 2 6 10 14 18 22 26
Cycle Day
Control 1
Control 2
Exercise 1
Exercise 2
Exercise 3
Exercise 4

-3.2 kg
-4.6 % Fat

Figure 7

## D-019 IGF-1



- 4.1 kg
- 5.2% Fat

Figure 8

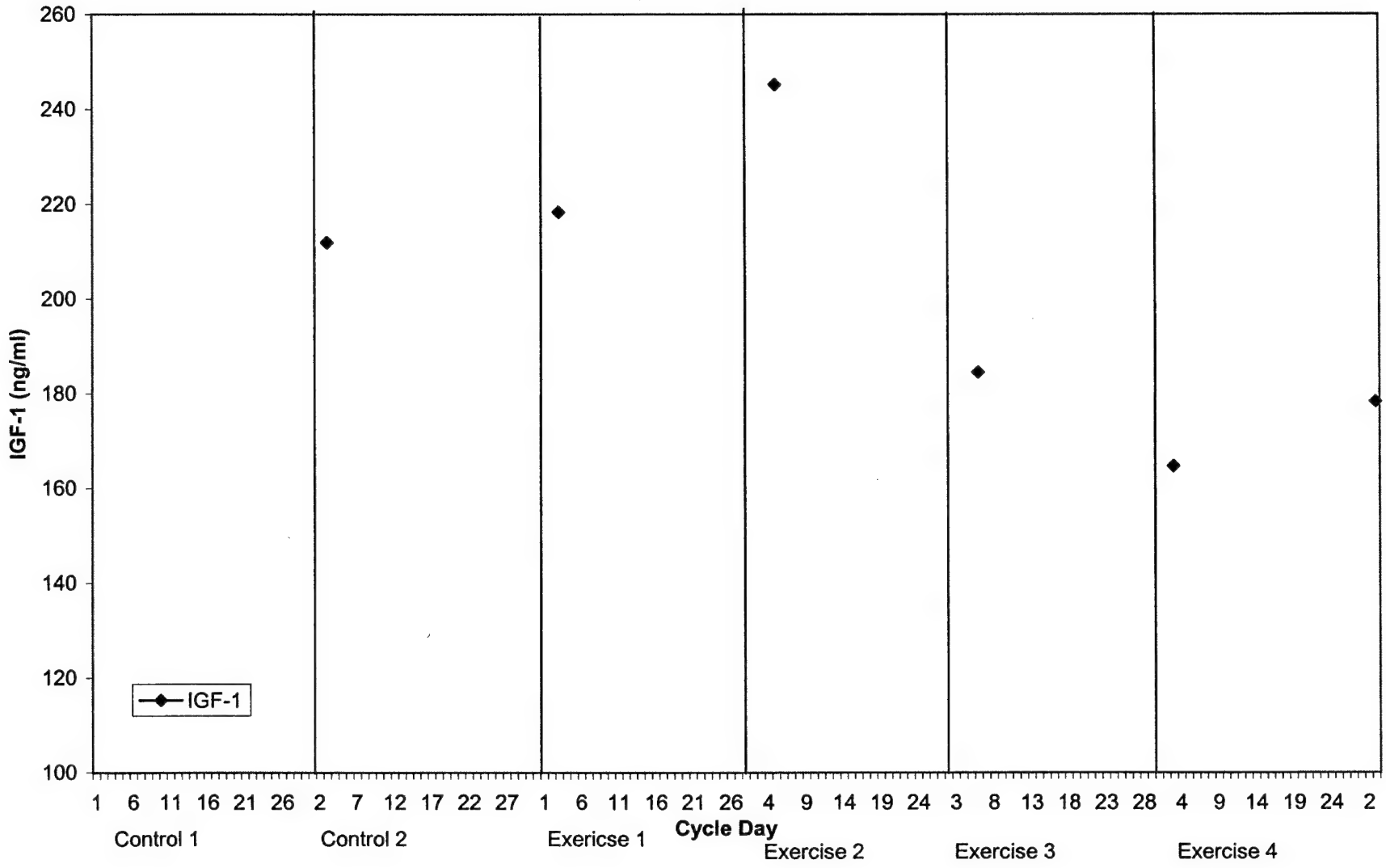
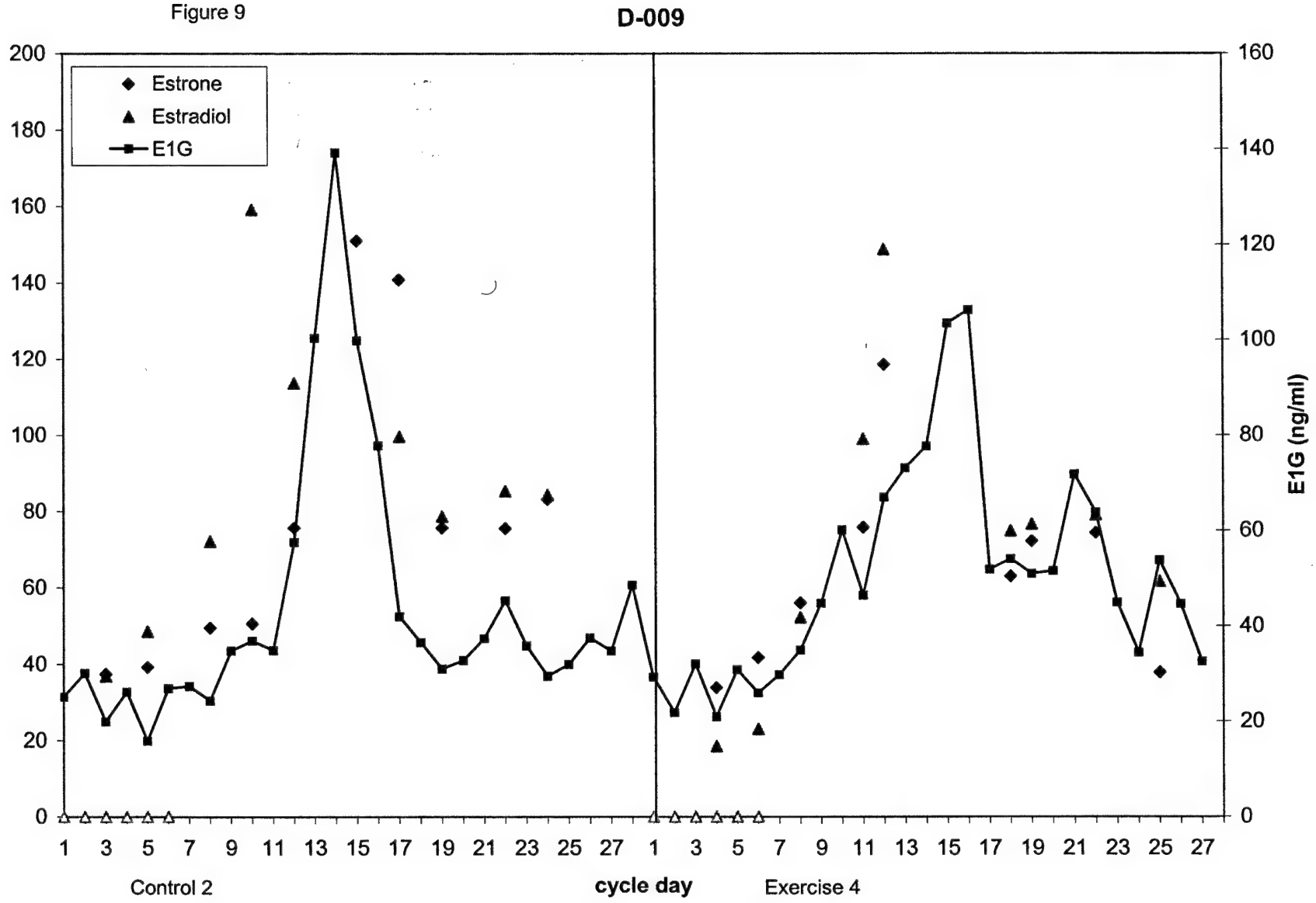


Figure 9


D-009
Estrone
Estradiol
E1G
200
180
160
140
120
100
80
60
40
20
0
160
140
120
100
80
60
40
20
0
E1G (ng/ml)
1 3 5 7 9 11 13 15 17 19 21 23 25 27 1 3 5 7 9 11 13 15 17 19 21 23 25 27
Control 2
cycle day
Exercise 4

Figrue 10

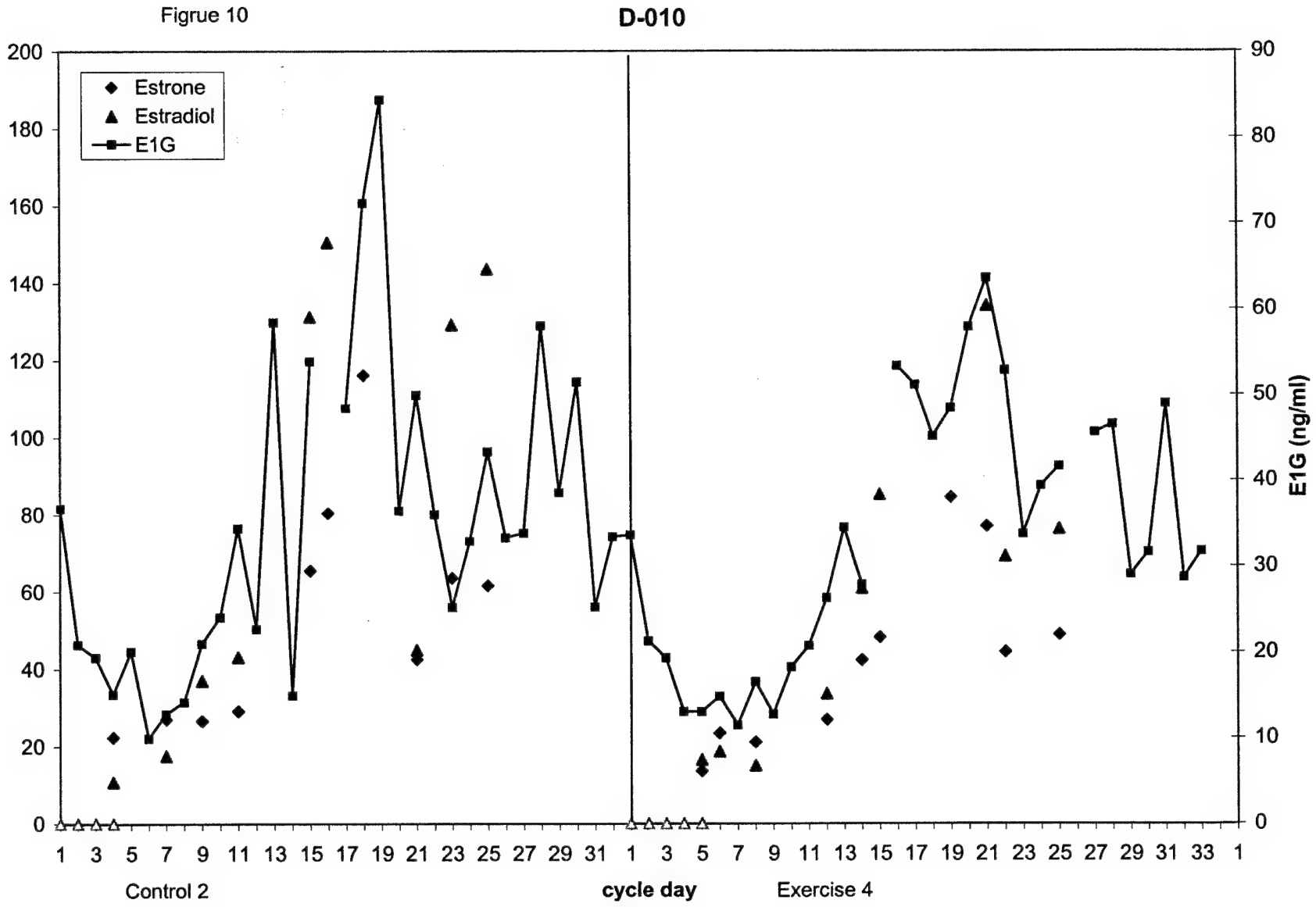

D-010
Estrone
Estradiol
E1G
200
180
160
140
120
100
80
60
40
20
0
90
80
70
60
50
40
30
20
10
0
E1G (ng/ml)
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 33 1
Control 2
cycle day
Exercise 4

Figure 11

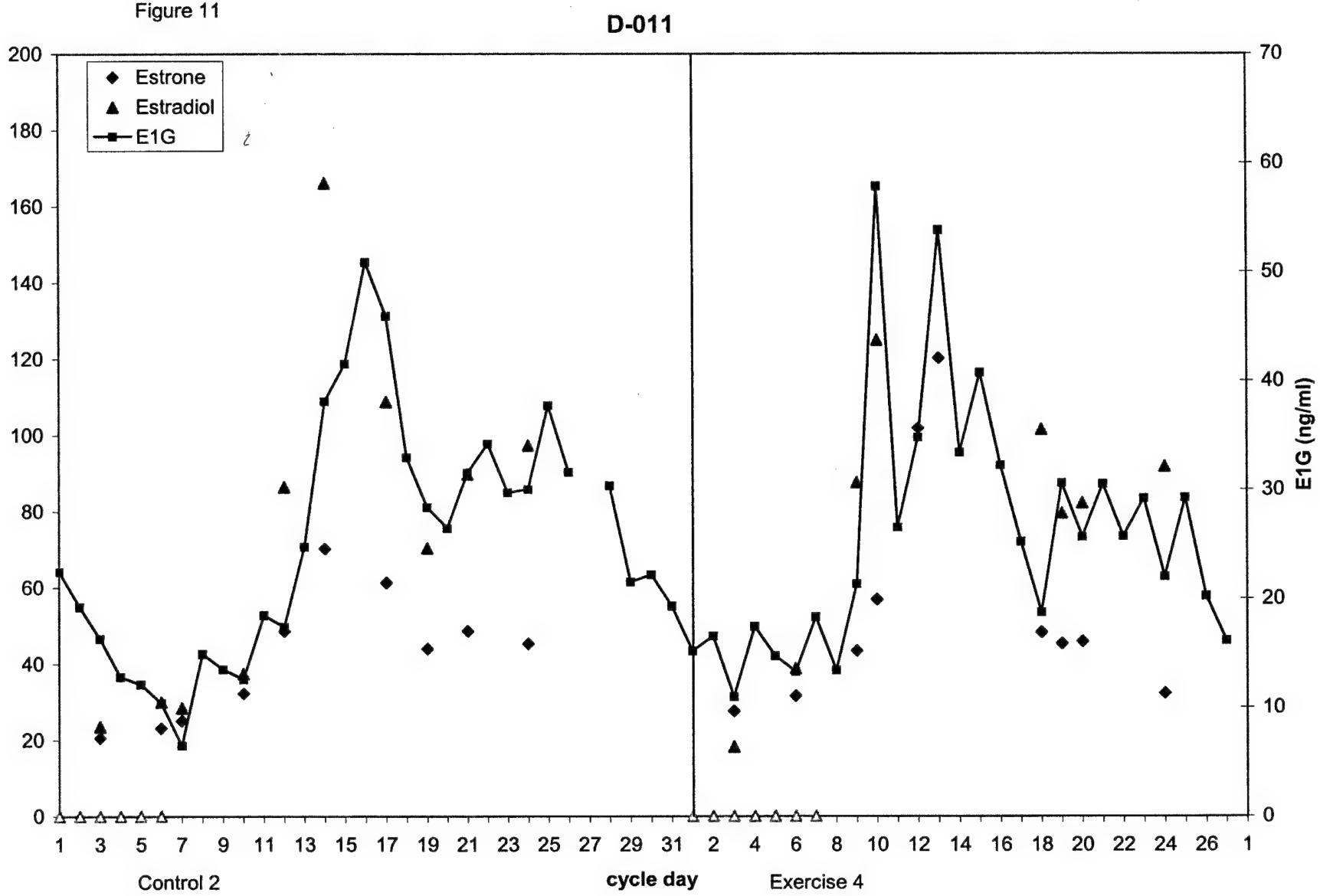
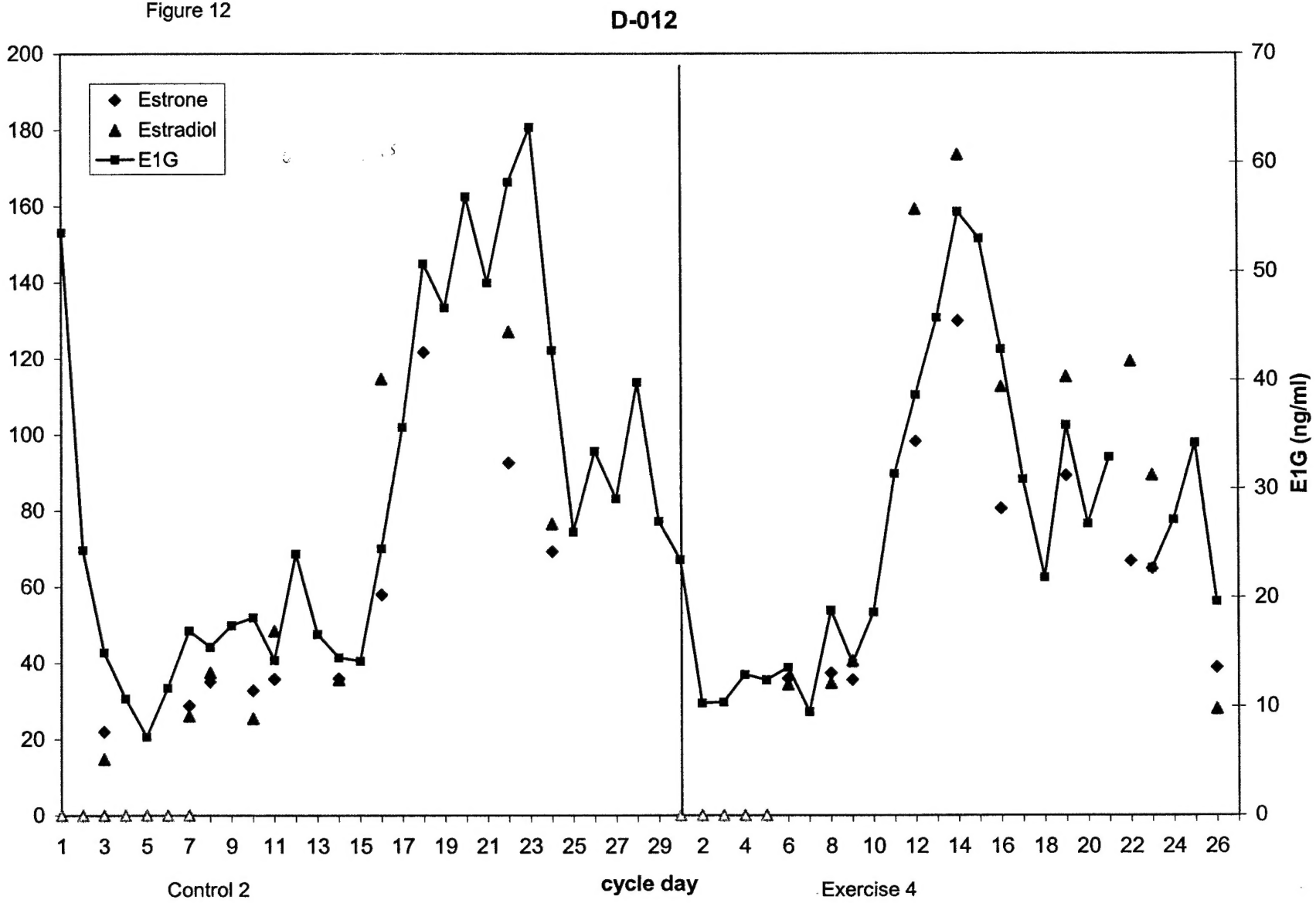

D-011
Estrone
Estradiol
E1G
200
180
160
140
120
100
80
60
40
20
0
70
60
50
40
30
20
10
0
E1G (ng/ml)
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 31 2 4 6 8 10 12 14 16 18 20 22 24 26 1
Control 2
cycle day
Exercise 4

Figure 12


D-012
Estrone
Estradiol
E1G
200
180
160
140
120
100
80
60
40
20
0
70
60
50
40
30
20
10
0
E1G (ng/ml)
1 3 5 7 9 11 13 15 17 19 21 23 25 27 29 2 4 6 8 10 12 14 16 18 20 22 24 26
Control 2
cycle day
Exercise 4

# Blood Spot Collection Protocol

As a sample collection technique, blood spot collection can offer certain advantages over other methods such as venipuncture, urine or saliva collection. Blood spots are relatively easy to procure, minimally invasive, and can be stored longer at room temperature than samples from other methods. The following describes a collection protocol designed to optimize the sample volume and standardize the sampling procedure.

1. The subject should wash her hands in warm water.

2. Bathe the hand in warm water for two minutes, or longer if necessary, to accomplish a flush of the skin.

3. Dry, and wipe the finger with an alcohol wipe.

4. Prick the finger with a lancet.

5. When blood flow begins, wipe away the first bit with a tissue.

6. The next drops are absorbed onto the paper (Schleicher and Schuell, #903) by positioning the finger just above, but not touching, the paper so that the blood wicks onto, rather than drops onto, the paper until the spot is large enough.

7. The paper should be handled by the edges only to avoid introducing any contaminating substances and placed on a clean surface or rack to air-dry at room temperature – usually about 4 hours, depending upon the humidity levels.

8. After drying, place the card in a zip-lock bag, preferably with a dessicant, and store at –20 C until analysis.

**Notes:**

Do not smear the blood onto the paper

Do not apply one drop onto another

Do not expose the spots to direct sunlight or other source of heat

Dr. Nancy I. Williams
Associate Professor
Room 267Q
Recreation Building
Department of Kinesiology
& Noll Physiological Research Center
Penn State University
University Park, PA 16802

Dear Dr. Williams,

At Dr. Granger's request, I am writing to outline the basic objectives for our development of blood spot assays for Total T3 and IGF-1 for your project. You should have received quotes for these projects from Martha Orland last weekend. As you are aware, we have already developed similar assays for testosterone, leptin, estradiol, and progesterone. Based on our previous experience I don't expect protocol development for these markers will be problematic. Nevertheless, as with any research project a specific timeline is difficult to predict. We hope the development work will take no longer than 3 months time.

As in the past, our approach will be to begin by modifying commerically available enzyme immunoassay protocols. The assay development will include determination of assay range, lower limit of sensitivity, linearity and spike recovery, and confirmation that intra- and inter-assay coefficients of variation are within acceptable limits outlined by Chard (1990). We will also provide recommendations regarding sample collection, preparation, and the amount of sample needed to perform each assay.

In a previous note to Dr. Granger, you mentioned having matched serum/plasma samples. Once the assay is internally validated we highly recommend comparing values from the blood spot assay protocols with results you obtain from the serum tests. We can arrange those serum tests for you if you don't already have a source for those assays.

Once completed, we can provide testing services for your project at a cost of $25.00 per sample for T3 and
>$30.00 per sample for IGF-1.

If you have any questions or are just interested in a progress report, please don't hesitate to call (800-790-2258 ext. 207) or email me (Ebs@salimetrics.com).

Best Regards,

Eve Schwartz
Director of Research and Development, Salimetrics LLC

August 20, 2003

Tech Support
Salimetrics, LLC
PO Box 395
State College, PA.
16804-0395

Re: Blood spot assays

Dear Technical Support Services:

We are currently running a study to test whether a program of moderate aerobic exercise that is combined with a moderate level of dietary restriction will result in significant decreases in two biomarkers of breast cancer. We also would like to validate a method of assessing energy status. We have been collecting dried blood spots from our volunteers. We wanted to know what blood spot assays you run. We would like to run some estrogen or progesterone assays. We would also like a cost estimate for supplies and running these assays on our blood spots. Currently, we have about 10 blood spots from our volunteers. We would appreciate any information that you could give us regarding your assays. We can be contacted through email at niw1@psu.edu or by calling our lab at 863-4488. Thank you in advance for your time and information.

Sincerely,

Nancy I. Williams
Primary Investigator